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14. ABSTRACT
Because of sports injuries, automobile accidents, falls, etc., and with the escalation of the use of improvised explosion devices (IEDs) by our enemies as witnessed in the most recent military conflicts in Iraq and Afghanistan, there has been an increased awareness of closed head concussions, also commonly referred to as the mild TBI (mTBI) injury. The prevalence of this type of closed-head brain injury, estimated as afflicting over 300,000 deployed soldiers or approximately 30% of all deployed troops, has distinguished it as the "signature injury" of these military conflicts. Despite the enormity of this medical problem, and recognition of the importance for the need to quickly and accurately diagnose the event in the face of a limited clinical presentation (i.e. no obvious wounds to the head), objective diagnostic tools and knowledge about what occurs in the brain following this type of injury are limited. Of equal concern is our lack of understanding the impact of multiple concussions on the brain and its consequences on the long term health of individuals. In order to address this problem, the WRAIR projectile concussive impact (PCI) model was developed under directive of the Combat Casualty Care Research Program (CCCRP). In this Year 1 Annual Report, we describe engineering advancements made to the PCI injury model including projectile impact energy/head kinematics and impact location. In addition, we describe the current results of studies focused on characterizing the neuropathologic, molecular and neurobehavioral changes following a single PCI.

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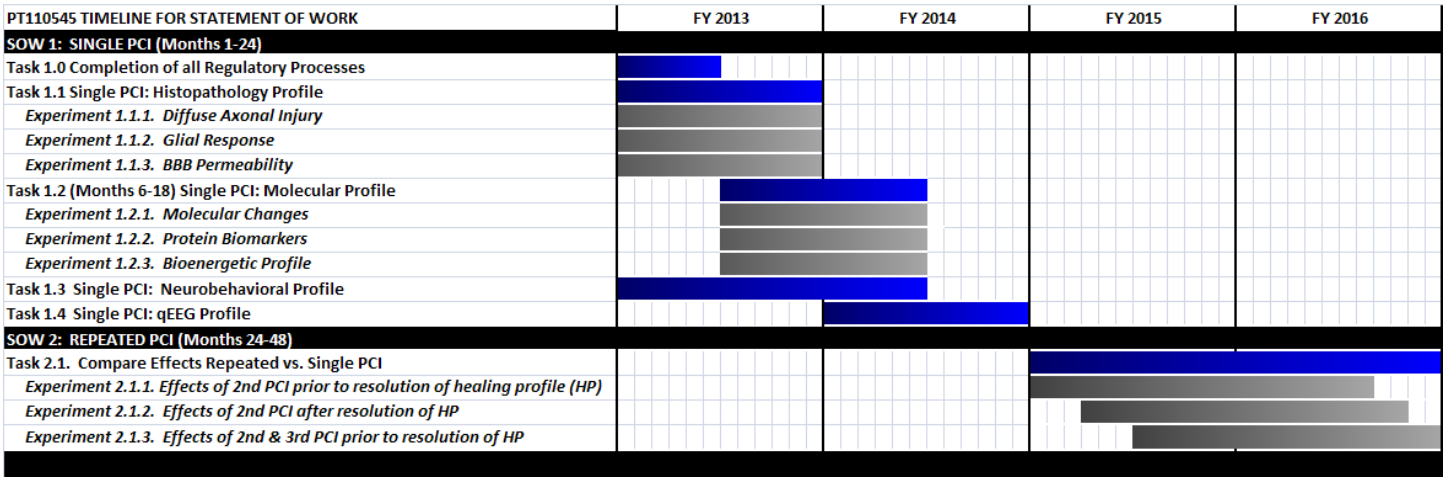
INTRODUCTION: Under the directive of the Combat Casualty Care Research Program (CCCRP), we completed the proof-of-concept development of a military-relevant model of closed-head concussive injury leading to mild traumatic brain injury (mTBI). In addition, in collaboration with the Composites and Hybrid Materials Branch, Army Research Laboratory (Aberdeen) we have recently completed the development and implementation of custom-designed helmets combined with pressure sensor film analysis; to detect the impact pressure distribution pattern both on the outer and inner helmet surface. The overall goal of the current proposal is to conduct longitudinal studies on the WRAIR projectile concussive impact (PCI) brain injury model following a "SINGLE" or "REPEATED" PCI injuries in order to develop a more thorough understanding of the changes taking place at a cellular level following a single or multiple concussive events, and to establish how those changes relate to clinically relevant mTBI behavioral and electrophysiological outcome metrics. Concussive head injury will be studied in the WRAIR PCI model using longitudinal and multi-modal designs to fully characterize the neuromotor, cognitive, emotional, and neuropathological evidence of brain injury. ***Phase I (SOW 1) studies will fully characterize the neuropathological, molecular and neurobehavioral changes following a "SINGLE" PCI injury. Phase II (SOW 2) studies will evaluate the cumulative effects of "REPEATED" PCI injuries based on outcome metrics defined in SOW 1.***



BODY

Task 1.0 (Months 1-6) Regulatory review and approval processing for studies involving animal subjects. The following animal protocols have been approved by the WRAIR IACUC: WRAIR IACUC Protocol # 12-PN-18S and 13-PN-30S. ACURO approval has been obtained for each of the study protocols. ***All regulatory review/approval requirements have been completed.***

During this timeframe, several engineering components of the PCI model have been refined to provide optimal injury parameters. The original PCI device used dry ice sublimation to build up pressure inside a microcentrifuge tube and trigger the release of a small projectile (i.e. the microcentrifuge cap) targeted to impact a helmet-protected rat head. However, we subsequently identified several limitations to the dry ice sublimation/microcentrifuge tube method and these limitations have been addressed by modifications made to (A) the PCI device and more recently to (B) the projectile. In addition, a pilot project was conducted to determine (C) the optimal angle of PCI injury. These modifications and results are summarized below:

(A) **PCI Device:** Started during the past year and completed during the 1st QTR of this proposal, the PCI device was modified to use compressed gas (i.e. nitrogen) instead of dry ice sublimation as the trigger mechanism for launching the projectile. In addition, a computer control interface was implemented to control the operating pressure (Figure 1). The primary advantage of using compressed gas vs. dry ice sublimation is that the mechanical forces used to induce the injury are far more controllable, reproducible and quantifiable. In addition, the "pressure wave" generated by the release of compressed gas is of low magnitude and is not related to the input pressure. Thus, the "pressure wave" effect has been eliminated. ***Moreover, the intensity of the force can be titrated to produce a wider spectrum***

of closed-head concussive injury severities for study. A patent application was submitted for this iteration of the device in August 2012 (U.S. Provisional Application Serial No. 61/521,446).

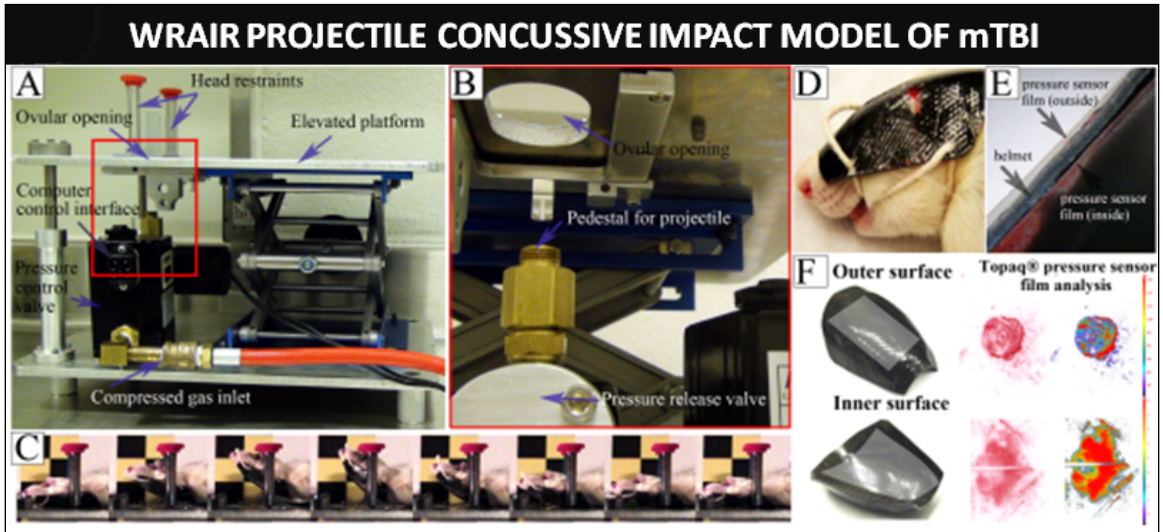


Figure 1. The WRAIR PCI Model of mTBI. (A) Elevated platform for holding the animal and aligning the projectile. (B) Instrument rig. (C) Head motion response to WRAIR PCI mTBI event. (D) Helmet made of woven glass-carbon. (E,F) Pressure sensor films are secured by adhesive to the inner and outer surfaces of the helmet. Pressure distribution is analyzed using the Topaq® system (Leung et al., 2012).

(B) **PCI Projectile**: In addition to intervals between repeated injuries; varying the intensity or severity of the mTBI insult is a critical factor to evaluate in preclinical mTBI studies (Fujito et al., 2012). In keeping with this, the modifications made to the PCI device also facilitate the use of small projectiles of different shapes/masses. Thus, during the 1st QTR of this project we collaborated with the Army Research Laboratory (ARL; Aberdeen MD) to test a number of small spherical (i.e. ball bearings) and cylindrical projectiles of different masses (ranging from 0.5 to 6g). ***The steel ball bearings have produced the most desirable and consistent pressure distribution profile on the inner surface of the helmet while remaining within a range that meets the criteria for mTBI.***

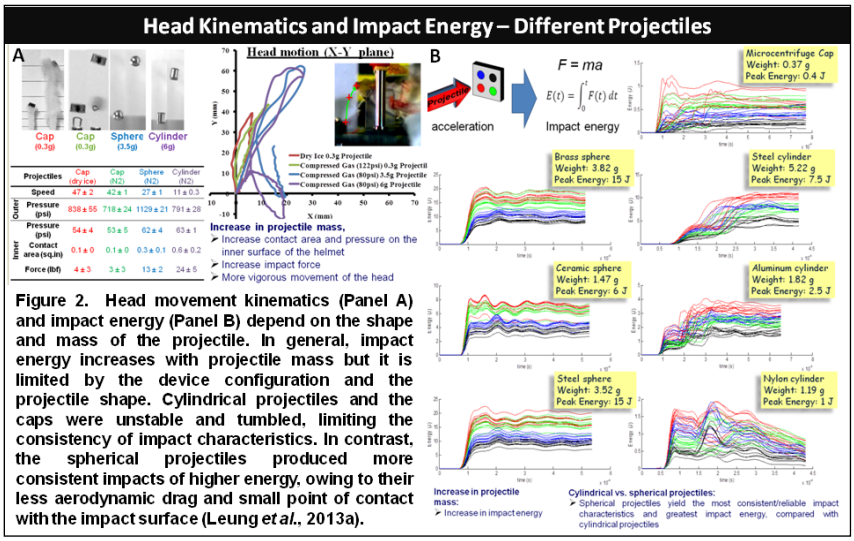


Figure 2. Head movement kinematics (Panel A) and impact energy (Panel B) depend on the shape and mass of the projectile. In general, impact energy increases with projectile mass but it is limited by the device configuration and the projectile shape. Cylindrical projectiles and the caps were unstable and tumbled, limiting the consistency of impact characteristics. In contrast, the spherical projectiles produced more consistent impacts of higher energy, owing to their less aerodynamic drag and small point of contact with the impact surface (Leung *et al.*, 2013a).

(C) **Angle of PCI Injury:** In an initial pilot experiment, we assessed PCI-induced injuries that were angled (A) 0° from the saggital plane (bilateral hit) or (B) either 45° or 90° from the saggital plane (unilateral hits). CatWalk automated gait analysis (Noldus, The Netherlands) was used to detect gait abnormalities at 2h, 1, 3, 7 days post-injury. Results showed that unilateral PCI produced a greater degree of gait alterations compared to bilateral PCI demonstrated by alterations in 46 or 32 (out of 210) gait parameters following the 45° and 90° hits respectively. In contrast, only 18 gait parameters were significantly altered following the bilateral (0°) PCI injury. Figure 3 provides a summary of the significant gait alterations detected in the three groups at different time points. Significant increases in mean

intensities of both front and hind paw prints were observed in rats subjected to unilateral hits (45° and 90°) at 1, 3 or 7 days post-injury ($p<.05$ vs. sham control). ***Collectively, the results indicate that unilateral PCI angled at 45° produces the most robust gait abnormalities that are sustained under repeated testing conditions.***

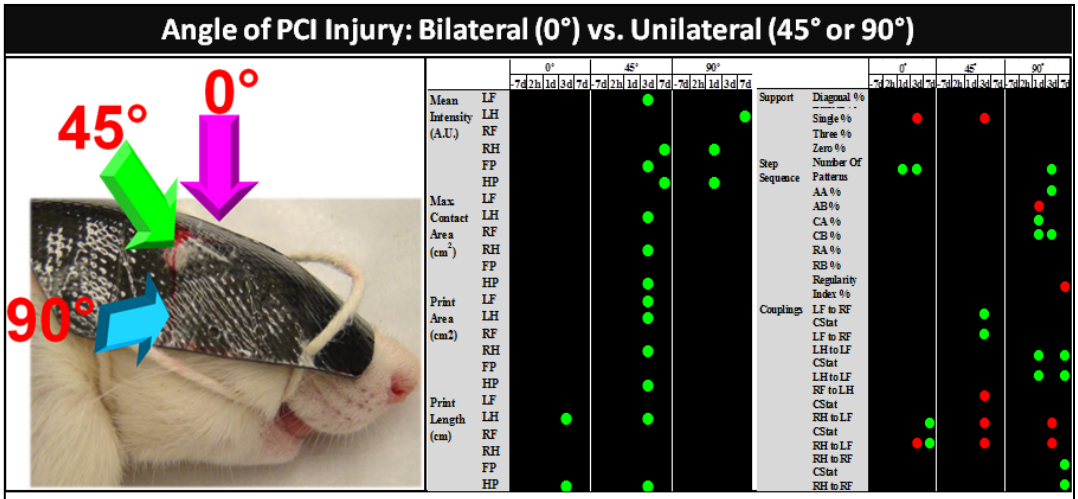


Figure 3. Adult rats were anesthetized with isoflurane, fitted with custom-designed helmets, and subjected to a single PCI angled at 0° (bilateral), 45° or 90° (unilateral) from sagittal plane. Sham controls received the same procedures without PCI. Gait performance was assessed using CatWalk automated gait analysis system at 7d prior to injury (baseline), 2h, 1d, 3d and 7d post-injury. Green (●) and red (●) dots represent significant increases or decreases vs. sham respectively (Leung et al., 2013a).

SUMMARY OF ADVANCED PCI MODEL: (1) the microcentrifuge cap in the original model has been replaced by a steel sphere (3.52 g) as the projectile; (2) pressure used to launch the projectile is set at 80 psi; and (3) the impact location is set at a 45° angle targeting the temporal-parietal region (right hemisphere). These advancements have been presented at the Society for Neurotrauma Symposium in Nashville TN (Leung et al., 2013) and are described in greater detail in Leung et al. (2013; manuscript in preparation). ***Importantly, all aspects and components of the refined/advanced PCI model have been approved in the current WRAIR IACUC Protocols 12-PN-18S and 13-PN-30S.***

PCI procedure (used for all tasks outlined below): The PCI injury apparatus consists of an elevated platform and a computer-controlled electro-pneumatic pressure release system used to launch a small projectile (3.52 g sphere) targeted at the rat's head. Following anesthetization with 5% isoflurane, a custom-designed helmet (Army Research Lab, Aberdeen Proving Ground, MD) is securely fastened onto the rat's head. Pressure sensor films (Fujifilm pre-scale pressure sensitive film) adhered to the inner and outer surfaces of the helmet are used to record the distribution and magnitude of pressure from the impact of the projectile. The anesthetized rat is placed on the elevated platform with its head positioned above an oval opening in the elevated platform such that the right hemisphere of the helmet-protected head is exposed to the projectile angled 45° from the saggital plane. A computer program is used to trigger the targeted release of the projectile at the rat's head. Immediately following PCI injury, the helmet is removed and the rat is returned to its home cage. Sham control animals receive the same procedures except the projectile impact.

It bears noting that the original study design called for the inclusion of a pressure wave (PW) control group to control for the potential effects of the PCI pressure wave. However, in the advance PCI system, the need for a "pressure wave" (PW) control group has been negated by the refinements made to advanced PCI system because the "pressure wave" generated by the release of compressed gas is minimal. As a substitute for the PW group, ***we have included a positive PCI control group in the experimental design when needed to confirm that the outcome measures are capable of detecting injury signals. For this purpose, animals***

were subjected to 4 PCI-induced concussions (1 hour apart), representing a more severe concussion, yet remaining within the limits of the mTBI spectrum.

PHASE 1 (Months 1-24): Fully characterize a "SINGLE" PCI head injury defining the acute temporal profile of histopathology, molecular (biomarkers/bioenergetics), neurobehavioral (motor/cognitive) dysfunction, and electrophysiological (EEG) changes following a single PCI. Control groups consist of animals that received anesthesia (sham) and animals exposed to repeated PCI (positive PCI control) only.

Task 1.1 (Months 1-12): Evaluate the regional and temporal profile of histopathological changes following a single PCI injury. The following experiments aim at elucidating diffuse axonal injury (DAI), glial response, blood brain barrier (BBB) permeability and brain edema using immunohistochemistry (IHC) at different time points and anatomical regions of the brain following PCI.

Experiment 1.1.1. Diffuse Axonal Injury (DAI): DAI is a hallmark pathologic feature of TBI and has been consistently detected across the spectrum of TBI severities, including mTBI. This study is focused on the expression of beta-amyloid precursor protein (βAPP) and amino cupric silver (CuAg) expression as markers for acute axonal injury. The effects of PCI on axonal injury using APP and CuAg staining assessed at 6h, 24h, 72h, 7d, 14d and 28d post-PCI.

Experiment 1.1.2. Glial Response: We previously reported significant increases in hippocampal expression of GFAP (glial fibrillary acidic protein; a marker for reactive astrocytes) in the PCI model at 24h post-injury. In the proposed study, the glial response to PCI injury are being examined in different brain regions (cerebral cortex, hippocampus, corpus callosum, thalamus, striatum and cerebellum) at 6h, 24h, 72h, 7d and 14d post-injury using immunostaining markers for reactive astrocytes and activated microglia. Following PCI injury, animals (n=8 per time point; 6h, 24h, 72h, 7d) were sacrificed with an overdose of ketamine/xylazine (70 and 6 mg/kg, i.m., respectively) and were transcardially perfused. Brains were harvested and post-fixed in 4% paraformaldehyde for 6 hours and then in 20% sucrose solution. The samples were processed at FD Neurotechnologies Inc. (Ellicott City, MD, USA). A series of coronal free floating brain sections (40 µm; 960 µm interval from +4.0 mm to -7.0 mm from Bregma) were immunostained for β-APP with Vector ABC system. Accumulation of β-APP in axons and axonal bulb formation indicate axonal damage. Another series of coronal sections was stained using FD NeuroSilverTM Kit II (FD Neurotechnologies Inc., Ellicott City, MD, USA) for neurodegeneration. For glial responses, a series of coronal brain sections was immunostained for glial fibrillary acidic protein (GFAP; astrocyte marker) and another series was immunostained for ionized calcium binding adaptor molecule (Iba1; microglia/macrophage marker). Images of the slides were digitized and examined using a BX61 microscope (Olympus, PA). Sham control groups (n=8 per time point; 24h and 7d) were included to control any possible effects of isoflurane anesthesia. A repeated PCI group (n=3) in which animals were subjected to 4 PCIs at 1h interval, was included at one acute post-injury time point only (1h following the final PCI injury) to serve as an initial positive control to assess the sensitivity of the outcome measures for detecting injury.

Qualitative examination of all stained tissue revealed positive β-APP staining indicative of β-APP accumulation and axonal bulb formation in the corpus callosum following both single and repeated PCI (Figure 4; top row). However, silver staining was negative in corpus callosum in all groups at the post-injury time points examined thus far (i.e. out to 7 days post-PCI). Both PCI and PCI positive control appeared to have more hypertrophied and reactive astrocytes (observed with GFAP) in the right hippocampus compared to the sham control. Similarly, hippocampal microglia exhibited a rounded amoeboid-like appearance and upregulated Iba-1 protein, suggesting an activation response following PCI (both single and positive control groups). Quantitative analyses of the histopathology profiles are ongoing and targeted for completion by January 2014.

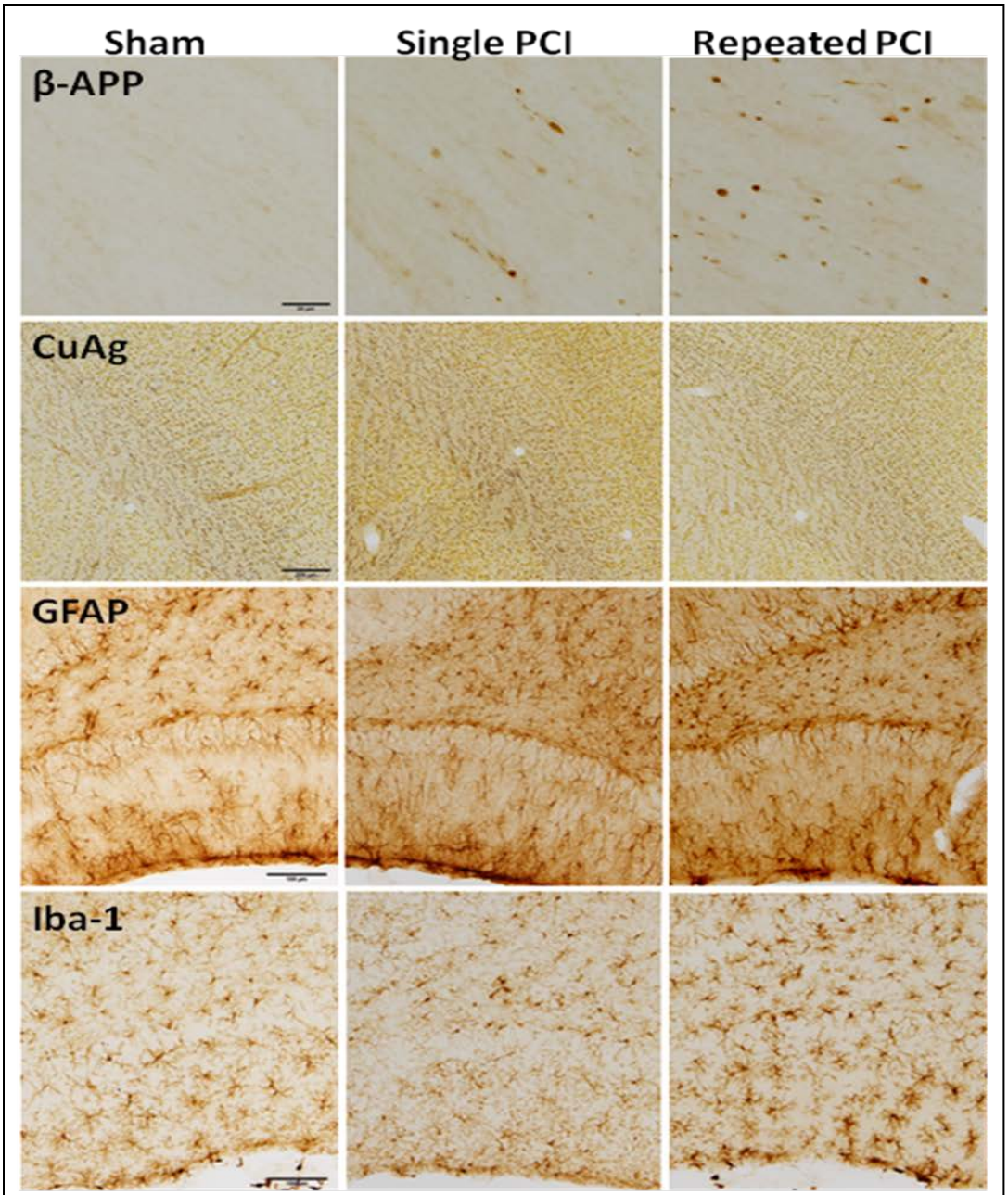


Figure 4. Representative images of brain sections stained with β-APP (first row; corpus callosum; 400X; scale bar: 20 µm); silver staining (2nd row; corpus callosum; 40x; scale bar: 200 µm); GFAP (3rd row; hippocampus; 100x; scale bar: 100 µm); and Iba-1 (4th row; hippocampus; 100x; scale bar: 100 µm).

Critically, thus far we have only analyzed tissue from repeated PCI (positive control) animals at 1 post-injury time point (i.e. 6h post-injury or 1h post the last PCI hit). Additional brains from repeated PCI controls (n=8/time point) at 24h, 72h, and 14 days post-injury are currently being collected for processing in order to confirm and/or assess the sensitivity of targeted histopathologic markers. Specifically, we plan to compare the effects of a single vs. repeated PCI (i.e. 4 hits; 1h intervals) on axonal (β-APP) and neuroinflammatory markers at the acute post-injury time points (i.e. 24h and 72h post-injury) and on both neuroinflammatory markers and neurodegenerative markers (i.e. silver staining) at 14 days post-injury.

Notably, axonal injury identified by β-APP accumulation and retraction balls (axonal swelling) have been observed in mild closed-head injury, both experimentally (Lewen et al., 1995) and clinically (Blumbergs et al., 1994) and has been associated with head acceleration/deceleration. In the PCI model, the rat head sustains a combined linear and angular acceleration (Smith et al., 2003). This suggests that the observation of acute increases in β-APP staining in the current study may be due to tissue deformation/axonal injury resulting from an intracerebral pressure gradient generated by linear acceleration and transient deformation of skull, as well as the shear and tensile strains generated by angular acceleration. ***Overall, the current findings that a single PCI injury results in significant, acute upregulation of β-APP positive staining provides further validation for this mTBI/concussion model.***

Experiment 1.1.3. Blood-Brain Barrier (BBB) Permeability: All tissue samples for BBB histopatholgy analysis have been collected for acute post-injury time points (i.e. 6h, 24h and 72h) using biotin dextran amine (BDA; 3 kDA). The involvement of astrocytes and/or tight junctions in the BBB breakdown process are being assessed by IHC using antibodies for (1) Aquaporin 4 (AQ4) co-labeled with GFAP, and (2) tight junction and endothelial linkage proteins occluden, zonula occluden 1, and claudin-5. Groups for Experiments 1.1.3 (n=8/group × 3 time points) consist of sham (one time point only), single PCI and repeated PCI (positive controls).

At the specified post-injury time points, animals were overdosed with ketamine/xylazine and monitored for distinguishing reflexes at 6, 24 or 72h post-PCI. The animals were then decapitated and the brain was rapidly removed and submerged in isopentane that had been super cooled with dry ice. The brains were wrapped in foil and stored at -80^{o}C freezer then shipped to FD Neurotechnologies Inc for processing and staining. Tissue processing and staining protocols have been optimized for fluorescent staining of AQ4 co-labeled with GFAP, zonula occluden-1 (ZO-1) and claudin-5 (Cld5), as shown in Figure 5.

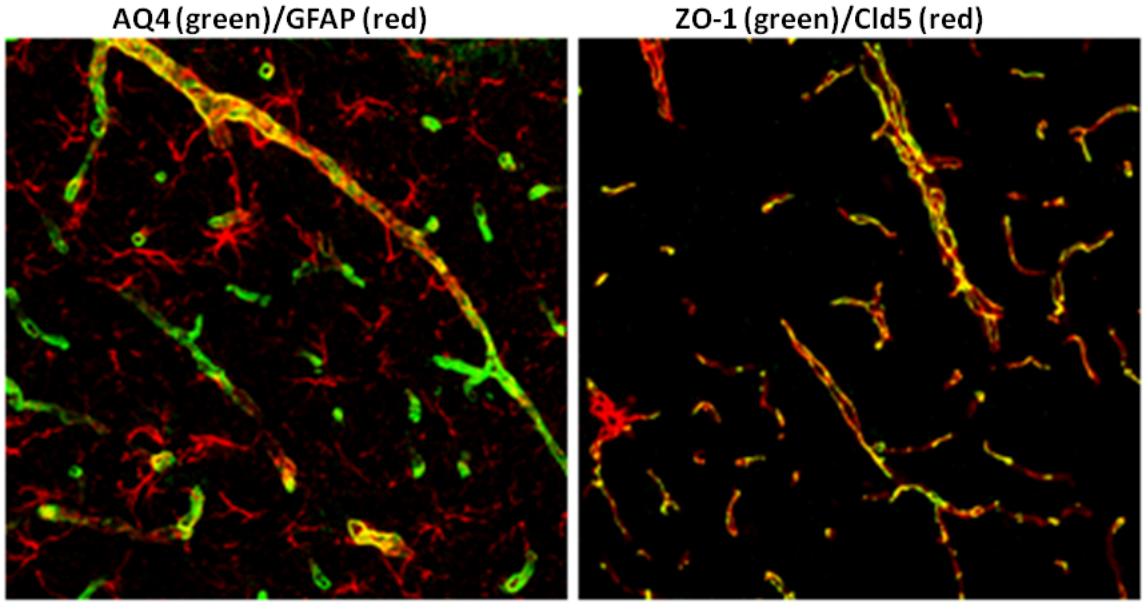


Figure 5. Representative images of brain sections stained with AQ4 co-labeled with GFAP (left) and ZO-1 with Cld5 (right).

Task 1.2 Regional and temporal profile of molecular/bionergetic changes following single PCI injury.

Experiment 1.2.1. mRNA Molecular Changes: All tissue has been collected for mRNA molecular analysis, including ipsilateral and contralateral tissue for five brain regions of interest (cortex, hippocampus, midbrain, hindbrain, and cerebellum) at 4 time-points (4 hr, 24 hr, 72 hr, and 7 days) and 2 groups (sham control and

single PCI) (n=10/grp).Total RNA isolation for cortex and hippocampus are 70% complete. Addition sample preparation continues. Depending upon results an additional time-point of 1 hr may be added. At the time of injury righting reflex data was collected on these animals and the latency to achieve normal righting reflex significantly increased following single PCI compared to sham controls.

Experiment 1.2.2. Protein Biomarkers: All tissue, serum and CSF has been collected for protein biomarker analysis, including ipsilateral and contralateral tissue for five brain regions of interest (cortex, hippocampus, midbrain, hindbrain, and cerebellum) at 5 time-points (1 hr, 4 hr, 24 hr, 72 hr, and 7 days) and 2 groups (sham control and single PCI) (n=10/grp). Total protein isolation for ipsilateral cortex and ipsilateral hippocampus tissue samples is complete. Addition sample preparation continues. In addition, positive control groups at the following conditions were included to account for the sensitivity of the outcome measures such as western blot for detecting injury: 3x (3 repeat PCIs) with a 2 hr interval, 3x with a 24 hr interval, 4x (4 repeat PCIs) with a 1 hr interval, and 4x with a 24 hr interval (n=5/group). Total protein isolation for positive controls is complete for all regions of interest.

Changes in protein abundance in GFAP and GFAP breakdown products have been evaluated in ipsilateral cortex and hippocampus at 4, 24, and 3 days post-single PCI by western blot. Briefly, tissue was homogenized in radioimmunoprecipitation buffer (RIPA) containing protease and phosphotase inhibitors. Total protein was normalized based on bicinchoninic acid (BCA) assay. Equal amount of total protein were run on polyacrylamide gels and transferred to PVDF membranes using the trans-blot turbo transfer system. Blots were blocked in 5% milk, probed with rabbit anti-GFAP primary antibodies overnight, washed, probed with donkey anti-rabbit secondary antibodies for 2 hours and detected using electrochemiluminescence. Blots were reprobed with anti-beta-actin antibody to control for protein loading. Analysis of band intensity was done using an LAS4000 and ImageQuantTL software (GE Healthcare). As a reference, moderate TBI models have shown increased GFAP expression in tissue starting at 1 and increasing at 3 days post injury.

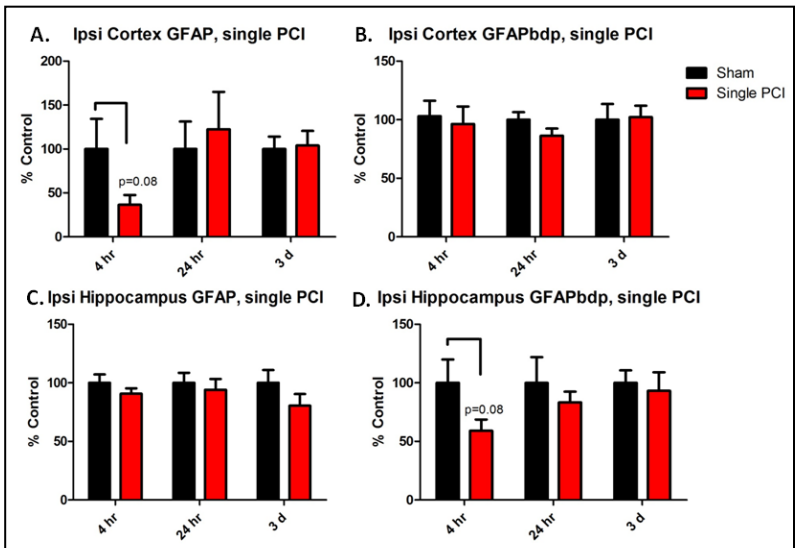


Figure 6. GFAP and GFAPbdp levels in (A,B) ipsilateral cortical tissue and in the (C,D) ipsilateral hippocampal tissue following single PCI. Protein levels from single PCI animals versus sham animals was evaluated at time points 4, 24 hr, 3 days post injury. The 50 kDa band corresponding to GFAP was measured. No significant alterations in GFAP or GFAPbdp expression were detected (error bars: standard error of the mean, n=9-10 per group).

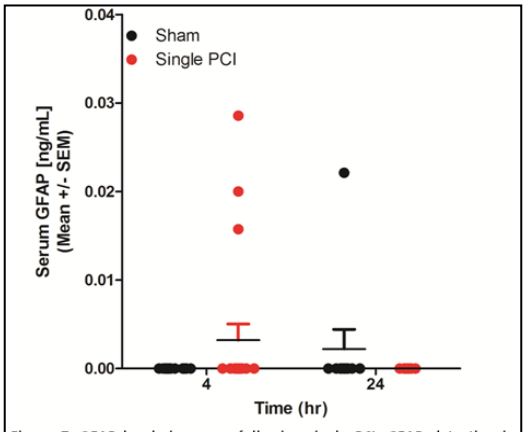


Figure 7. GFAP levels in serum following single PCI. GFAP detection in serum isolated after sham (black circles) or single PCI (red circles), was analyzed by colorimetric ELISA. The individual replicates and mean +/- SEM of total GFAP (ng/mL) is displayed for sham (4hr n= 10; 24hr n = 5, black circles) and single PCI (4hr n= 10; 24hr n = 5, red circles).

Following single PCI no significant changes were detected in GFAP or GFAPbdp from 4 hr through 3 days in ipsilateral cortex or ipsilateral hippocampus (Figure 6A-D). However, GFAP appeared to be decreased in cortical tissue at 4 hr, suggesting an acute post-injury loss of cortical astrocytes (Figure 6A). In contrast, reduced GFAPbdp expression in the hippocampus at 4 hr post-injury suggests a transient reduction in GFAP turnover in this brain region (Figure 6D). Additional evaluation of the effects of repeated PCI on GFAP, GFAP breakdown products is currently in progress.

Total GFAP was also evaluated in serum following a single PCI injury (Figure 7) using Enzyme Linked Immunosorbent Assays (ELISAs). Briefly, plates were coated overnight with GFAP capture antibody (Banyan Biomarkers) and then blocked for 30 min at room temperature. After washing, 100µL of serum was added in duplicate and incubated for 2 h at room temperature. Next, plates were

incubated with GFAP detection antibody for 2 h at room temperature. Signal was detected with substrate and measured using a colorimetric plate reader (450nm). GFAP quantification was determined from a standard curve. Detection and accuracy was confirmed with internal calibrator controls and the ELISA has been validated using more severe models of TBI such as penetrating ballistic-like brain injury (PBBI). GFAP ELISA data was analyzed by 1-tailed t-Tests between individual groups per time point. Data analyzed by 1-sample t-Test to determine variation from a theoretical mean of "0" (i.e. not detectible), which is the expected value for Sham. ***Overall, no significant effect of PCI on GFAP levels was detected in serum or in brain tissue samples between 4 hr to 72 hr post-injury. Based on human data which shows increased serum GFAP levels at acute post-injury time points following concussion/mTBI we plan to evaluate GFAP in serum and brain tissue at 1 hr post injury following both single and repeated PCI.***

Experiment 1.2.3. Bioenergetic Profile: Changes in metabolic activity levels are being assessed following a single PCI injury using the electromagnetic tissue fixation method to prepare brains for ultra-performance liquid chromatography (UPLC) measurements of adenosine triphosphate (ATP), adenosine diphosphate (ADP), creatine, phosphocreatine and N-acetylaspartate (NAA) levels to establish a profile of metabolic vulnerability/recovery in the PCI model (n=8/grp × 3 groups × 5 time points; N = 120). Cerebral metabolic activity was maximally preserved using microwave fixation method during euthanasia. Microwave-fixed brain tissues were collected at 2h, 6h, 24h, 72h and 7 days following a single PCI (n=8 per time point). Samples from sham control were collected at 2h and 24h (n=8 per time point). Samples from the PCI positive control (4 PCIs at 1h interval) were collected at 2h only. Samples from the 2h time point are being processed by Metabolon Inc to generate global metabolic profiles based on a library of over 4,000 known mTBI-related metabolites.

Task 1.3 (Months 1-18). Evaluate the neurobehavioral (motor, cognitive, and affective) profile following PCI injury.

Experiment 1.3.1 Gait assessment: Animals were randomized between four cohorts: sham (anesthesia alone), single PCI, repeated PCI positive control (4 PCIs at 1h interval) and repeated sham (rsham – to control for repeated anesthesia effect). Gait assessment was conducted 3 days prior to PCI (baseline measures) and at 2 h, 24 h, 72h, 9 d, 28 d, and 3 months post-injury using the CatWalk automated gait analysis system (Noldus Information Technology Inc., Leesburg, VA). The CatWalk is a highly sensitive device consisting of a 1.3 m long glass plate illuminated on the side by dim fluorescent lighting. In a dark (unlit) room, animals are placed on the walkway and allowed to traverse from one end to the other. Direct contact between the paw and glass surface results in light reflection in the form of illuminated footprints. Footprint images are video-recorded by a camera positioned under the walkway. Sensorimotor abnormalities following PCI were assessed using the CatWalk at the indicated post-injury time points. Thus far, we have completed testing at 2 – 72 hr post-injury and results indicated that a ***significant number of gait parameters were altered following either a single PCI or repeated PCI (Table 1). Moreover, whereas gait abnormalities following a single PCI appeared more transient (resolving by 24h post-injury); gait disturbances following repeated PCI were more pronounced by 72h post-PCI (Figure 8).***

Table 1. Injury-Induced Alterations in Gait Parameters

GROUP	TIME POST-INJURY		
	2 h	24 h	72 h
Single PCI	12	3	4
Repeated (4x) PCI	16	11	58

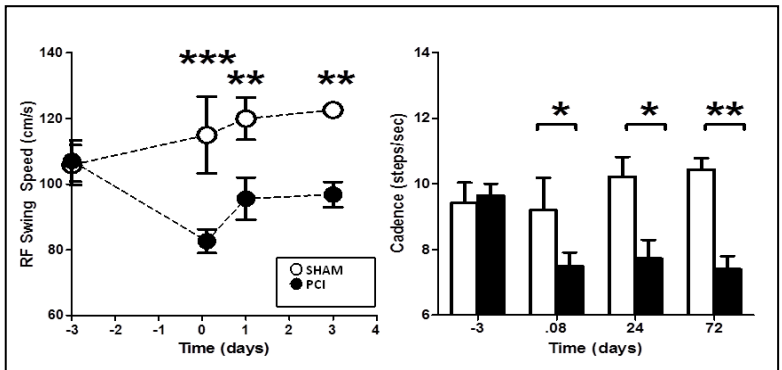


Figure 8. Changes in select gait parameters following ***repeated*** PCI (positive control group; 4 PCIs at 1 h interval). (A) Changes in the swing speed of the right front (RF); (B) Changes in the number of steps per second (cadence) * p <0.05; **p < 0.01, *** p < 0.001.

Experiment 1.3.3. Cognitive assessment: To validate the sensitivity of the cognitive assessment in the PCI model, rats were randomly assigned into two groups: sham and repeated PCI. The sham control group received a single anesthesia (4% isoflurane), whereas the PCI positive control group received four PCIs at 1 hour intervals. At 72 hours post-injury, all rats were subjected to a five-day working memory test using Morris water maze. Rats were tested in a circular pool (75cm deep and 175cm in diameter) filled with clear water. The position of the platform changed each day rotating within the four quadrants (Northeast, Northwest, Southeast and Southwest). The rats were placed in the pool at the same starting position (i.e. West) throughout the experiment. Each rat received 4 trials per day for 5 consecutive days. Each trial lasted 60 seconds with an inter-trial interval (ITI) for 5 minutes. The latency to find the platform was recorded and used for statistical analysis. ***The working memory version of the MWM task failed to detect a deficit between the sham and the PCI positive control group at the acute post-injury time point (Figure 9). Further studies evaluating cognitive function using different variations of this task and the novel object recognition (NOR) task at both acute and chronic time points are ongoing.***

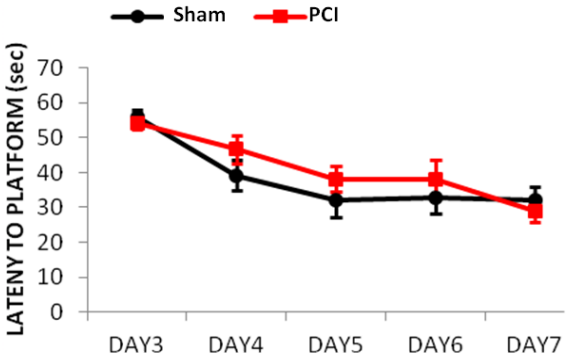


Figure 9. Morris water maze assessment of working memory at 3-7 days post-injury. The latency to platform was recorded over 5 consecutive days. N=12 per group. All data was expressed as mean ± standard error (SEM).

Righting Reflex Assessments: In both clinical and experimental TBI, the loss of righting reflex indicates loss of consciousness (LOC) that is associated with the injury severity (Dewitt et al., 2013). Based on this new information, we have measured the righting reflex of animals across all the experiments and have summarized those results in Table 2 and Figure 10. In general, loss of righting reflex of 15-30 min in animal TBI models is considered moderate-severe TBI, and <15 min is considered mild (Dewitt et al., 2013). ***In the PCI model, significant increases in time to return of righting reflex ($p<.05$ vs. sham) were observed following a single PCI injury. Further, the righting reflex recovery time remained significantly higher than controls after each consecutive PCI injury, but not in sham animals exposed to repeated anesthesia (Figure 10). Finally and perhaps most critically, the righting reflex suppression ranged from 6 to 9 min thereby meeting the 'mild' criteria even following repeated PCI (i.e. positive control groups).***

Table 2. Summary of Righting Reflex Data

		Task 1.1	Task 1.2	Task 1.3	
		Histopathology	Molecular profiles	Neurobehavioral profiles	
			Proteomics/ Genomics	MWM	Gait
Sham (anesthesia)	1st	222.5 ± 36.7	233.1 ± 11.1	184.8 ± 19.8	223.1 ± 28.7
	2nd	-	-	-	402.0 ± 62.5
	3rd	-	-	-	348.6 ± 51.3
	4th	-	-	-	374.8 ± 61.4
PCI	1st	348.3 ± 34.0	414.5 ± 19.79	256.8 ± 27.7	433.4 ± 43.6
	2nd	367.4 ± 60.1	-	528.2 ± 41.8	714.1 ± 68.8
	3rd	415.0 ± 67.1	-	489.5 ± 64.4	733.9 ± 136.1
	4th	397.7 ± 85.4	-	486.8 ± 52.2	518.8 ± 73.4

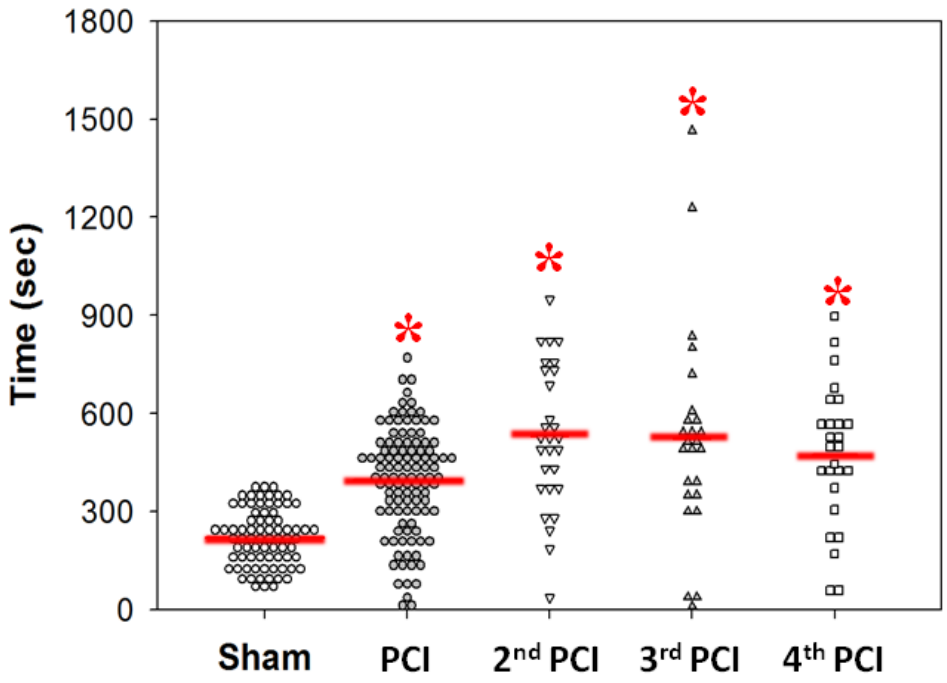


Figure 10. Recovery time of righting reflex of sham control, single PCI and repeated PCI (positive control (4 hits, 1h apart) and repeated sham control. *p<0.05 compared to sham control (one-way ANOVA with Dunn's post-hoc tests)

RESEARCH ACCOMPLISHMENTS:

1. IACUC and ACURO approval completed for two active protocols (in vivo and molecular).
2. Completion of Advanced PCI Model and Parameters to include PCI Device (driven by compressed gas vs. dry ice sublimation); completion of helmet material and design testing; PCI projectile; angle/location of PCI injury on the rat head.
3. Completion of acute (6h – 7 days post-injury) histopathological studies of a single PCI. Analysis of chronic post-injury time points is in process.
4. Completed collection of all histopathological samples for evaluating blood brain barrier (BBB) permeability following a single PCI. Brain tissues are being processed and analysis is targeted for completion in the first Quarter of Year 2.
5. Completed all tissue collections for mRNA molecular and protein biomarker changes. Analysis of the effects of PCI on GFAP and GFAP breakdown products has been completed. Analysis of additional markers is ongoing.
6. Completed sample collection for changes in metabolic activity levels. Primary (2h) samples are currently being processed via contractual agreement by Metabolon for global analysis of over 4,000 metabolites.
7. Completed acute post-injury assessment of motor (i.e. gaitwalk) abnormalities following a single PCI. Chronic evaluations are ongoing.
8. Completed acute post-injury assessment of cognitive (MWM) function following a single PCI. Additional animals are currently being tested at chronic time points.
9. Added righting reflex measures to the neurobehavioral outcome parameters and reported results confirming the validity of the PCI model as a model of closed-head concussive mild TBI.

REPORTABLE OUTCOMES: The following abstracts were presented during Year 1 of this research effort.

Leung LY, Larimore Z, Holmes L, Cartagena C, McLoughlin S, Bustos F, Schmid K, Shear DA, Tortella FC. The WRAIR Projectile Concussive Impact Model: Effects of Impact Direction and Projectile Property. Military Health System Research Symposium 2013. Fort Lauderdale, Florida. August 2013. (Oral presentation)

Leung LY, Larimore Z, Holmes L, Cartagena C, McLoughlin S, Bustos F, Schmid K, Shear DA, Tortella FC. The WRAIR Projectile Concussive Impact Model: Effects of Impact Direction and Projectile Property. The 31st National Neurotrauma Symposium. Nashville, Tennessee, USA. August 2013.

CONCLUSION: The initial, proof-of-concept, PCI injury device utilized a simple design that consisted of heating a small, torque-sealed, microcentrifuge tube packed with dry ice to launch a targeted projectile (microcentrifuge cap) and utilized a prototype stainless steel helmet to prevent skull fracture and subdural hematoma (Chen et al., 2012). However, the original PCI model had several limitations including: 1) the stainless steel helmet was too thick and rigid and left empty, irregular gaps between the helmet and the rats head, 1) the prototype helmet was also too heavy with respect to the weight of the rats head, and it thus restricted rotational head motion upon impact, 3) the impact energy was limited by the microcentrifuge tube and sublimation of the dry ice such that 4) neither the impact force nor the projectile velocity could be titrated (i.e. it was an all-or-none effect regardless of the amount of dry ice packed into the tube). Therefore, a second generation device was developed that utilizes compressed nitrogen to launch a projectile (Figure 1). The primary advantage of using compressed gas vs. dry ice sublimation is that the mechanical forces used to induce the injury are far more controllable, reproducible and quantifiable. In addition, several modifications were made to the engineering components in the PCI model including (1) custom fabricated helmet material construction and design and (2) the testing of different PCI projectiles. Additionally, a pilot study was conducted to determine the optimal angle/location of the impact to the head.

With these modifications, the WRAIR PCI model was refined to provide a consistent and highly reproducible concussion in rats. Experimental results demonstrating clinically relevant signs such as LOC, gait disturbances and axonal injury are included in this report. Overall the current results confirm that the PCI model creates a highly reproducible head impact injury across subjects that is truly non-invasive and requires no surgical procedures. As such it provides a valuable tool for studying the injury mechanism of closed-head impacts, particularly repeated concussion. In addition, by virtue of its high throughput (12 animals can be injured per hr), simple design and relative ease of fabrication, the model will provide an optimal exploratory platform for future diagnostic and therapeutic preclinical studies that can readily be implemented in other research laboratories.

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